

# Field response of near-isogenic brown midrib sorghum lines to fusarium stalk rot, and response of wildtype lines to controlled water deficit

D. L. Funnell-Harris<sup>ab\*</sup> , P. M. O'Neill<sup>ab</sup> and S. E. Sattler<sup>ac</sup>

<sup>a</sup>United States Department of Agriculture, Agricultural Research Service, Lincoln, NE; <sup>b</sup>Department of Plant Pathology, University of Nebraska, Lincoln, NE; and <sup>c</sup>Department of Agronomy and Horticulture, University of Nebraska, Lincoln, NE, USA

To increase digestibility for ruminant livestock and for lignocellulosic ethanol conversion efficiency in sorghum (*Sorghum bicolor*), brown midrib (*bmr*) lines carrying *bmr6* or *bmr12* and the double mutant (*bmr6 bmr12*) in two backgrounds (RTx430 and Wheatland) were developed, resulting in lines with significantly reduced lignin, as compared with the near-isogenic wildtype. Under greenhouse conditions, these lines had previously demonstrated no increased susceptibility, and some lines were more resistant to the highly virulent stalk rot pathogen, *Fusarium thapsinum*, compared to the wildtype. Fusarium stalk rot of sorghum is a destructive disease that under high temperatures or drought conditions may lead to lodging. To determine if greenhouse observations could be extended to field environments, *bmr* and near-isogenic wildtype lines were inoculated with *F. thapsinum* at field locations, Mead (irrigated) and Havelock (dryland) in Nebraska, USA. Analysis of mean lesion lengths showed those of most *bmr* lines were statistically similar to the wildtype. Across both genetic backgrounds, *bmr6* and *bmr6 bmr12* double mutant plants grown at Mead had significantly smaller mean lesion lengths than the corresponding wildtype ( $P \leq 0.05$ ). To assess responses of the two genetic backgrounds to controlled (greenhouse) water stress, wildtype RTx430 and Wheatland plants were inoculated with *F. thapsinum* under well-watered and water stress conditions. Mean lesion lengths resulting on water deficit plants were significantly larger than those on well-watered plants ( $P = 0.01$ ). These results indicate that this bioassay can be used to screen sorghum lines in the greenhouse for increased resistance or tolerance to both drought and fusarium stalk rot.

**Keywords:** brown midrib, field experiment, *Fusarium thapsinum*, *Sorghum bicolor*, stalk diseases, water stress

## Introduction

Sorghum (*Sorghum bicolor*) is a highly versatile crop with different cultivars bred for grain, forage or syrup production. This C<sub>4</sub> crop is drought tolerant and can be grown in areas with limited water resources. Recently, sorghum has drawn considerable attention as a biofuels crop for use in grain-based ethanol production and for potential in lignocellulosic biofuels production (Nghiem *et al.*, 2016).

Stalk diseases are significant constraints for sorghum production worldwide (Jardine & Leslie, 1992; Tesso *et al.*, 2010). Such diseases can result in lodging, which poses difficulties in mechanical harvesting, thus reducing biomass yield. Additionally, forage quality can be decreased due to deterioration of stalks. Stalk diseases can also affect grain yields, which may be due to stunting of plant growth, to impaired ability of stalks to translocate water, nutrients or photosynthates to

developing grain, and to reduced grain harvest from lodged plants (Bandara *et al.*, 2017).

The current study focused on fusarium stalk rot, which is caused by several *Fusarium* species (Tesso *et al.*, 2010). Fusarium stalk rot is typified by deterioration of the cortical parenchyma cells surrounding the vascular bundles (Bandara *et al.*, 2017). The pathogens can grow endophytically (without symptoms) through the plant (Funnell-Harris *et al.*, 2010) but disease can be manifested following drought conditions during flowering or early grain fill (Tesso *et al.*, 2004).

Disease control methods that can mitigate effects of environmental conditions, such as irrigation, may reduce stalk rot. However, sorghum is commonly grown on marginal lands under rain-fed conditions, in part due to its endogenous drought-tolerance, and therefore irrigation to avoid stalk diseases is impractical. In sorghum, resistance to fusarium stalk rot has been difficult to identify because several *Fusarium* spp. may act as causative agents. In addition, resistance to stalk rot is a quantitative trait profoundly affected by environment (Bramel-Cox *et al.*, 1988; Jardine & Leslie, 1992; Tesso *et al.*, 2004). However, one trait that is associated with increased stalk rot and drought tolerance is delayed post-flowering senescence, called ‘stay-green’ (Borrell *et al.*,

\*E-mail: deanna.funnell-harris@ars.usda.gov

2014). As the name implies, plants with this trait maintain green leaves longer under stress than non-stay-green varieties (Burke *et al.*, 2010). Despite concerted efforts to breed for increased drought tolerance in sorghum, many commonly used sorghum breeding lines, cultivars and hybrids lack the stay-green trait.

Responses of brown midrib (*bmr*) sorghum lines have demonstrated resistance to some stalk pathogens in greenhouse studies (Funnell-Harris *et al.*, 2010, 2014, 2017a). The *bmr* phenotype is characterized by discoloration of the leaf midrib. These plants have mutations in monolignol biosynthesis, the pathway that produces the subunits that are incorporated into the lignin polymer (Sattler *et al.*, 2012). Two well-characterized mutations, *bmr6* and *bmr12*, resulted in noticeable reductions in lignin and improved digestibility for livestock feed and cellulosic ethanol conversion as compared with wildtype lines (Dien *et al.*, 2009). The double mutant lines had even further reductions in lignin, increased digestibility, and increased ethanol yields as compared to either *bmr6*, *bmr12* or the wildtype lines (Dien *et al.*, 2009; Sattler *et al.*, 2010). Although lignin has been traditionally accepted as important in inducible defences against or as a barrier to pathogens (Nicholson & Hammerschmidt, 1992), changes in monolignol biosynthesis result in changes to other phenylpropanoid compounds in cell walls, which may result in plants with no increased susceptibility or plants with increased resistance to pathogens as compared to their wildtype counterparts (Funnell-Harris *et al.*, 2010; Gallego-Giraldo *et al.*, 2011; Tesso & Ejeta, 2011). This increased resistance may in part be due to an increase in phenylpropanoids within the cell wall (Palmer *et al.*, 2008), which may directly inhibit pathogens or may act indirectly to induce other defence pathways (Salzman *et al.*, 2005; Funnell-Harris *et al.*, 2014, 2017a).

Greenhouse inoculation studies with *Fusarium* spp. were previously performed on *bmr* plants and near-isogenic wildtype plants, using wound inoculations of either the peduncle or the base of the stalk, because natural infection of these tissues can lead to lodging of the grain head or the entire plant, respectively (Bramel-Cox *et al.*, 1988). In no case was the *bmr* line more susceptible to any given pathogen than the near-isogenic wildtype line. Peduncle inoculations with *Fusarium thapsinum*, *Fusarium verticillioides* and *Fusarium proliferatum* demonstrated increased resistance in *bmr* lines (as measured by lesion length; Funnell-Harris *et al.*, 2010, 2017a); however, plant genetic background affected response of lines with these mutations. When basal stalk inoculations were performed with these pathogens, there were no significant differences in mean lesion lengths or they were significantly smaller than those on the corresponding wildtype (*F. proliferatum*) (Funnell-Harris *et al.*, 2014, 2017a). All of these experiments were performed under optimal greenhouse conditions and plants were well watered.

Observations made under controlled greenhouse conditions may not always reflect what occurs in the field (Diaz

Arias *et al.*, 2013). Therefore, it was necessary to determine whether the responses of *bmr* lines, which were as resistant, or more resistant, to *F. thapsinum* than near-isogenic wildtype in the greenhouse, were similar in the field. In the current study, near-isogenic *bmr* lines (*bmr6*, *bmr12* and double mutants *bmr6 bmr12*) in two backgrounds (Pedersen *et al.*, 2006, 2008) were grown along with wildtype lines in fields, one irrigated and the other dryland, at two locations in Nebraska, USA, then inoculated with *F. thapsinum*. Also in this study, the wildtype genetic backgrounds of the *bmr* lines were assessed in a greenhouse assay, in which inoculation with *F. thapsinum* was conducted under water-deficit stress, applied in a controlled manner, as compared with inoculated plants under well-watered condition. The hypotheses tested were: (i) *bmr* lines are not more susceptible to fusarium stalk rot (*F. thapsinum*) inoculation in field studies; and (ii) fusarium stalk rot disease development under drought stress conducted under greenhouse conditions will not result in increased susceptibility to the pathogen.

## Materials and methods

### Plant lines and fungal isolates

Near-isogenic sorghum lines *bmr6* and *bmr12*, and the *bmr6 bmr12* double mutant in the genetic backgrounds RTx430 and Wheatland were previously developed and maintained by USDA-ARS, Lincoln, NE (Pedersen *et al.*, 2006, 2008). Grain for field and greenhouse experiments was produced in the greenhouse.

The *F. thapsinum* isolates used in the field study were H03S-11-9, which was isolated from grain grown at Havelock field in Lincoln, NE, and M05A\_1J\_3b, which was collected from a spore trap at Mead, NE. Isolate H03S-11-9, previously known to have high virulence on sorghum stalks (Funnell-Harris *et al.*, 2014), was used for inoculations at Havelock. For the current study, a screen was performed using wound inoculations of peduncles (Jardine & Leslie, 1992) to compare the isolate obtained from Mead, M05A\_1J\_3b, with H03S-11-9 before using in field inoculations at Mead. The resulting mean lesion lengths (M05A\_1J\_3b: 60.3 ± 33.5 mm; H03S-11-9: 74.7 ± 33.5 mm) were not significantly different ( $P = 0.76$ ).

Isolates were maintained in long-term storage on potato dextrose agar (PDA) slants (stored at 4 °C), in glycerol stocks (−80 °C), and in silica gel-skimmed milk stocks (4 °C). For working cultures, fungi were transferred to one-half strength PDA, prepared using full-strength potato dextrose broth (PDB; Becton Dickinson and Co.) and amended with 100 µM ampicillin (Sigma-Aldrich). Fungal inoculum for stalk inoculations was prepared by adding an agar disk (5 mm in diameter) from the edge of 4-day-old PDA cultures per 5 mL of sterile PDB with sterile toothpicks, which were previously treated to remove toxins and other inhibitors of fungal growth (Jardine & Leslie, 1992; Funnell-Harris *et al.*, 2010, 2014). The broth-and-toothpick cultures were incubated for 10 days at 22 °C before inoculations.

### Field experiment

The field experiment was conducted at two locations in eastern Nebraska: Havelock field at University of Nebraska, Lincoln

(lat 40.8609, long -96.5951; 347 m a.s.l.), and University of Nebraska's Eastern Nebraska Research and Extension Center near Mead, NE (lat 41.1637, long -96.4088; 351 m a.s.l.), during 2014, 2015 and 2016. Eastern Nebraska has a humid continental climate with wet springs, warm to hot summers and mild to cold and relatively dry autumns. Table S1 lists mean monthly temperatures, total monthly rainfall and supplemental irrigation (Mead only) during sorghum-growing months of May to October. Weather data was recorded by the High Plains Regional Climate Center, University of Nebraska (<https://hprcc.unl.edu/>).

During 2014, 2015 and 2016, nitrogen fertilizer was applied at 112 kg ha<sup>-1</sup> before planting both fields. During 2014 at Mead and Havelock, atrazine [6-chloro-*n*-ethyl-*N'*-(1-methyl-ethyl)-1,3,5-triazine-2,4-diamine] was applied at 1.1 kg ha<sup>-1</sup> immediately after planting for weed control. At Mead, this was followed by applications of alachlor [2-chloro-2',6'-diethyl-*N*-(methoxymethyl) acetanilide] at 4.75 L ha<sup>-1</sup> approximately 1 week post-emergence and quinclorac (3,7-dichloro-8-quinolinecarboxylic acid) and atrazine at 0.37 and 1.1 kg ha<sup>-1</sup>, respectively, 3 weeks post-emergence. At Mead, supplemental irrigation was applied using overhead sprinklers on 15 July, 21 July and 15 August (38.1 mm per application) to avoid water stress (Table S1). During 2015 at Havelock, atrazine was applied (1.1 kg ha<sup>-1</sup>) about 1 week after planting, then chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] was applied 6 weeks after planting for grasshopper control. At Mead, atrazine (1.1 kg ha<sup>-1</sup>) was applied immediately after planting then approximately 14 days after emergence alachlor (4.75 L ha<sup>-1</sup>) was applied. At Mead, supplemental irrigation was applied (25.4 mm) on 22 July and 26 July. During 2016 at both fields, atrazine (1.1 kg ha<sup>-1</sup>) was applied immediately after planting. At Mead, supplemental irrigation (25.4 mm) was applied on 16 June and 28 July.

Details of planting, inoculation and lesion measurement dates are in Table S2. For each year, plots consisted of two 7.6-m rows spaced 76 cm apart with 120 seeds planted per row. As previously reported (Pedersen *et al.*, 2008), emergence of the Wheatland double mutant *bmr6 bmr12* is significantly less than the wildtype, while emergences of all three RTx430 near-isogenic *bmr* lines are significantly less than the wildtype. Plots had been previously planted with soybean before each year at Havelock, and soybean before the first 2 years and sorghum before the third year at Mead. During 2014, the plant densities of Wheatland lines, and to a lesser extent RTx430, were reduced due to additional application of the herbicide fomesafen to those plots in the previous year. Five plants within each plot were randomly chosen and marked with red loop-lock tags (27.9 × 2.5 cm; Gempler's) and numbered 1 to 5 for pathogen inoculations, and five plants were similarly marked and labelled with blue tags for control inoculations. Plants within 0.75 m of each end of the rows, or near tracks left by irrigation equipment, were not inoculated. The pathogen inoculation was the *F. thapsinum* isolate from the same field (H03S-11-9 for Havelock inoculations and M05A\_1J\_3b for Mead inoculations) prepared as 'broth-and-toothpick' cultures as described in the previous section. The control inoculation was also a wound inoculation but the toothpicks were incubated in sterile PDB; this inoculation indicates the extent of the highly pigmented wound response common in sorghum. Within each two row plot, one row had three plants with *F. thapsinum* inoculum and two plants with the broth control, and the second row had two *F. thapsinum* and three control inoculations, with 30–50 cm of uninoculated plants between each inoculated plant; plants with different inoculations were interspersed within a row.

Plants were inoculated during anthesis (defined as half the anthers exerted in half the plants, 57–83 days after planting; Table S2). A surface-disinfected awl was used to form a shallow hole in the stalk at the second internode above the roots, then a fungus- or sterile broth-incubated toothpick was inserted into the small hole. Between 32 and 38 days after inoculation (dai; Table S2), depending on weather, irrigation or other delays, each plant was cut at ground level, the stalk was split longitudinally, and the length of the resulting red to purple discoloration resulting from wounding and pathogen ingress (defined as the lesion) was measured using a clear plastic metric ruler, as previously described (Funnell-Harris *et al.*, 2014; Bandara *et al.*, 2017). The total length measurement could include discoloration that nearly spanned the diameter of the stalk and/or discoloration in a narrow line.

A split-plot design was used for the field experiment, with sorghum genetic background (Wheatland or RTx430) defined as the whole-plot and *bmr* line (wildtype, *bmr6*, *bmr12* or *bmr6 bmr12*) as the split-plot randomized within each background. Five plants per split-plot were randomly selected for each inoculum treatment (fungus or control). The experiment was replicated over time (years) at each location. The number of plants per line (8) per treatment (fungus or control) per year (2014, 2015 and 2016) at each location was 5 for a total of 15 plants per line and treatment and 240 plants total for each location. The data were analysed using PROC MIXED procedure of SAS/STAT software (SAS, 2013). At each location, year was considered a fixed effect to determine whether year-to-year differences in weather or field treatments could affect responses to inoculum. Genetic background, *bmr* line and inoculum were also considered fixed effects. Datasets for each location were analysed for Levene's homogeneity of variance and appropriate adjustments were incorporated using the REPEATED/GROUP option. Least squares means (LSM) and standard errors (SE) are reported. Differences of LSM were considered significant at  $P \leq 0.05$ .

### Greenhouse water deficit experiment

In the field study, RTx430 and Wheatland background plants could not be directly compared between the two field environments (dryland and irrigated) because of the requirement to use different pathogen isolates obtained from each location. Therefore, a greenhouse assay was designed in which wildtype plants were differentially watered, and responses to a given *F. thapsinum* isolate under different watering regimes could be directly compared. To assess the effects of sorghum genetic background under controlled well-watered or water deficit conditions to *F. thapsinum*, wildtype plants of cultivars Wheatland and RTx430 were compared. Assays were conducted essentially as previously reported (Funnell-Harris *et al.*, 2014) with the addition of differential water treatments beginning 67 days after planting (dap). Greenhouse-grown grain from each of the two cultivars were sown into pasteurized standard soil mix (one part sand, one part coarse vermiculite, one part top soil, and two parts shredded peat moss) in 25.4-cm-diameter pots. Seedlings were culled to one plant per pot. Plants were initially watered daily for 67 dap (approximately 2 weeks before anthesis), then water was applied daily to one-half of the plants in order to maintain levels similar to field capacity (well-watered). The other half of the plants were allowed to dry until soil moisture content was below 25% field capacity, defined herein as water deficit, before water was applied. For all plants, soil water content was monitored three times per week using a 10HS Moisture Sensor (Decagon Devices) with a U30 Shuttle (Hobo). Soil water

content was determined approximately 24 h after previous watering of well-watered plants, then all plants with the well-watered treatment (daily watering), and water deficit plants with soil water content below 25% field capacity (intermittent watering), were watered. The base of the stalk of each plant was inoculated at anthesis, as described in the previous section, approximately 2 weeks after initiating differential water treatments. Lesion length measurement was conducted 32 dai (Funnell-Harris *et al.*, 2014). Four repetitions were conducted throughout 2 years: the first was planted on 26 June 2014, inoculated on 19 August 2014, and harvested on 19 September 2014; the second was planted on 18 December 2014, inoculated on 2 March 2015, and harvested on 3 April 2015; the third was planted on 20 April 2015, inoculated on 22 June 2015, and harvested on 24 July 2015; and the fourth was planted on 7 December 2015, inoculated on 29 February 2016, and harvested on 31 March 2016.

The greenhouse experiment was conducted as a split-plot design with water treatment (well-watered or water deficit) serving as the whole-plot and genotype (Wheatland or RTx430) as the split-plot. There were eight replicates, blocked by location in a greenhouse, and four repetitions of the entire experiment conducted over time. Analyses for mean lesion length and soil moisture levels were performed. Replication within a repetition was treated as a random effect. Assay, cultivar, water treatment and inoculum were all considered fixed effects. The data were analysed using PROC MIXED procedure of SAS/STAT software (SAS, 2013). Datasets were analysed for Levene's homogeneity of variance and appropriate adjustments were incorporated using the REPEATED/GROUP option. LSM and SE are reported. Differences of LSM were considered significant at  $P \leq 0.05$ .

Pearson correlations were generated for the response variables lesion length and moisture content for pathogen and control inocula, and pathogen-only inoculum and drought-stressed plants using the PROC CORR in SAS/STAT software (SAS, 2013).

## Results

### Response of *bmr* lines to *F. thapsinum* under field conditions

Because different *F. thapsinum* isolates were used at each location, albeit with similar tested levels of virulence, and plant culture conditions were different (e.g. supplemental irrigation at Mead but not at Havelock), the locations were examined separately. For the field at Havelock, the effect of year was significant ( $P < 0.01$ ) but not plant genetic background or *bmr* genotype ( $P \geq 0.31$ ). For the field at Mead, the effect of genetic background was significant ( $P = 0.05$ ) but not year or *bmr* genotype ( $P \geq 0.11$ ). At both locations, the effect of inoculum (*F. thapsinum* versus control) was significant. Genetic background  $\times$  inoculum interactions were also significant at both locations ( $P \leq 0.01$ ).

Across both genetic backgrounds at Mead, mean lesion length following *F. thapsinum* inoculations on *bmr* lines was similar (*bmr12*;  $P = 0.54$ ) or significantly smaller (*bmr6* plants and *bmr6 bmr12* double mutant;  $P \leq 0.05$ ) than on the wildtype (Table 1). Across both genetic backgrounds at Havelock, mean lesion lengths following inoculations with *F. thapsinum* on *bmr* lines were similar to those on wildtype plants ( $P \geq 0.15$ ).

**Table 1** Mean lesion lengths (mm) resulting from basal stalk wound inoculations with *Fusarium thapsinum* of brown midrib (*bmr6*), *bmr12* and *bmr6 bmr12* double mutant lines and near-isogenic wildtype lines, across two plant genetic backgrounds, in the field at two locations, Mead and Havelock, NE, USA, in 2014, 2015 and 2016.

Location <sup>a</sup>	Inoculum <sup>b</sup>	Plant genotype <sup>c</sup>	Mean lesion length (mm) <sup>d</sup>
Mead	<i>F. thapsinum</i>	Wildtype	56.8 $\pm$ 3.8 a
		<i>bmr6</i>	44.8 $\pm$ 3.9 bcd
		<i>bmr12</i>	53.6 $\pm$ 3.8 ab
		<i>bmr6 bmr12</i>	46.7 $\pm$ 3.9 b
	Control	Wildtype	33.3 $\pm$ 2.9 cde
		<i>bmr6</i>	37.4 $\pm$ 2.8 cd
		<i>bmr12</i>	30.5 $\pm$ 2.8 de
		<i>bmr6 bmr12</i>	28.0 $\pm$ 2.8 e
Havelock	<i>F. thapsinum</i>	Wildtype	54.0 $\pm$ 4.8 a
		<i>bmr6</i>	50.9 $\pm$ 4.7 a
		<i>bmr12</i>	46.0 $\pm$ 4.7 a
		<i>bmr6 bmr12</i>	44.3 $\pm$ 4.7 a
	Control	Wildtype	23.8 $\pm$ 2.0 bc
		<i>bmr6</i>	22.8 $\pm$ 2.0 c
		<i>bmr12</i>	28.4 $\pm$ 2.0 b
		<i>bmr6 bmr12</i>	22.7 $\pm$ 2.0 c

<sup>a</sup>Mead: University of Nebraska's Eastern Nebraska Research and Extension Center near Mead, NE, USA; fields at Mead were irrigated with overhead sprinklers. Havelock: University of Nebraska, Lincoln, NE, USA; Havelock field was dryland.

<sup>b</sup>The pathogen inoculation was an *F. thapsinum* isolate from the same field. The control inoculation was toothpicks incubated in sterile broth.

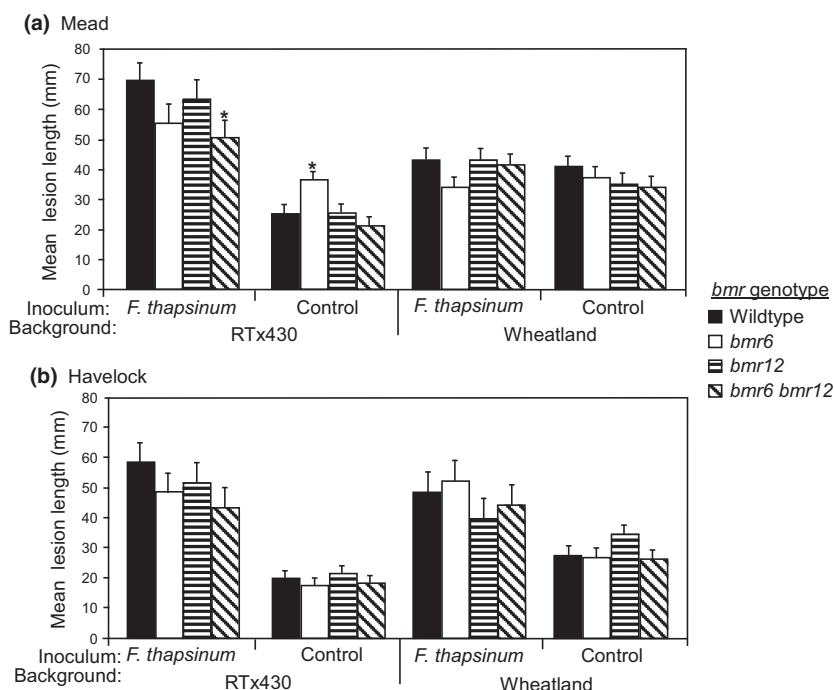
<sup>c</sup>Wildtype, *bmr6*, *bmr12* and *bmr6 bmr12* double mutant near-isogenic lines were in the genetic backgrounds Wheatland and RTx430.

<sup>d</sup>Least squares means and standard errors are reported. At each location, values with different letters are significantly different at  $P \leq 0.05$ .

When RTx430 and its near-isogenic *bmr* plants grown at Mead were inoculated with *F. thapsinum*, mean lesion length on single mutant lines (*bmr6* or *bmr12*) was similar to that of the wildtype ( $P \geq 0.10$ ), while this value on the *bmr6 bmr12* double mutant was significantly different ( $P = 0.03$ ; Fig. 1). For RTx430 plants at Havelock, mean lesion lengths for *bmr* lines were similar to the wildtype ( $P \geq 0.10$ ). For Wheatland plants grown at both locations and inoculated with *F. thapsinum*, mean lesion lengths on *bmr6*, *bmr12* and *bmr6 bmr12* double mutant lines were similar (at Mead  $P \geq 0.11$ ; at Havelock  $P \geq 0.35$ ) to those of the wildtype.

With regard to plant genetic background, mean lesion lengths resulting on RTx430 and Wheatland following inoculations with *F. thapsinum* inoculations at Mead were significantly different ( $P < 0.01$ ), but not at Havelock ( $P = 0.34$ ). At Mead, for all *bmr* genotypes, mean lesion lengths on Wheatland plants were significantly smaller than those on RTx430 plants for the wildtype, *bmr6* and *bmr12* ( $P \leq 0.01$ ), but not for *bmr6 bmr12* double mutant plants ( $P = 0.29$ ; Fig. 1).

Most control inoculations (to determine wound responses) at Havelock resulted in a mean lesion length that was significantly different from the pathogen inoculation in the same line ( $P \leq 0.02$ ), except for Wheatland



**Figure 1** Response of field-grown brown midrib (*bmr*)6, *bmr12* and *bmr6 bmr12* double mutant sorghum plants to the fusarium stalk rot pathogen *Fusarium thapsinum*. Near-isogenic plants in two genetic backgrounds, RTx430 and Wheatland, were grown over 3 years at two locations (a) Mead, and (b) Havelock. Plants were inoculated with *F. thapsinum* or sterile broth (control inoculations). Lesion lengths (mm) were measured, 32 to 37 days after inoculation. Least squares means (LSM) and standard errors (SE) are reported. Bars represent LSM; positive SE are shown. Asterisks indicate that the mean lesion length resulting on the *bmr* line is significantly different from that on the corresponding wildtype line with the same inoculum ( $P \leq 0.05$ ).

*bmr12*, in which it was similar ( $P = 0.52$ ; Fig. 1). At Mead, all control inoculations of RTx430 lines resulted in mean lesion lengths significantly different from the pathogen inoculations ( $P \leq 0.01$ ); however, control inoculations of *bmr6* resulted in a response significantly greater than the control inoculation on wildtype plants ( $P = 0.01$ ; Fig. 1). At Mead, control inoculations of Wheatland plants resulted in responses consistently similar to lesions resulting from *F. thapsinum* on corresponding *bmr* genotypes ( $P \geq 0.17$ ).

#### Response of sorghum to stalk rot under differential water treatments in greenhouse conditions

Water deficit and well-watered conditions were applied differentially to plants from 2 weeks before anthesis, through inoculations during anthesis, and until scoring of lesions, 32 dai. For the water deficit treatment, soil moisture was maintained at approximately 25% field capacity, and for the well-watered plants, soil moisture was maintained at field capacity. Figure 2 shows the range of responses following stalk inoculations with *F. thapsinum* or the broth control under water deficit and well-watered conditions. In water deficit conditions (Fig. 2a), the response of a RTx430 plant following inoculation with *F. thapsinum* resulted in a thin line of dark pigmentation, indicating that the pathogen apparently grew upward along a vascular bundle. In contrast, the response of a Wheatland plant showed a broad necrotic lesion that was apparently narrowed and reduced at a node. In well-watered plants, tissues lacking visible symptoms appeared to be healthier than stalks under water deficit conditions, but this was not always

observed. Figure 2b indicates the wound response following inoculation with toothpicks incubated in sterile PDB under water deficit or well-watered conditions.

For analysis of mean lesion length, there was an effect of assay, water treatment and inoculum ( $P \leq 0.01$ ), as expected, but not for cultivar or its interactions with water treatment or the three-way interaction with inoculum and water treatment ( $P \geq 0.23$ ); however, cultivar  $\times$  inoculum interactions were significant ( $P < 0.01$ ). Across cultivars and all four assays, mean lesion length resulting from inoculation with *F. thapsinum* under the well-watered condition was significantly smaller than when a water deficit regime was applied ( $P = 0.01$ ; Fig. 3a). Mean lesion length resulting from *F. thapsinum* inoculation across all assays in a single cultivar was not significantly different between the water deficit and well-watered treatments (RTx430:  $P = 0.06$ ; Wheatland:  $P = 0.07$ ). In individual assays, RTx430 (assay 1) or Wheatland (assay 2) inoculated under water deficit condition had significantly larger mean lesion length than that resulting on plants inoculated under well-watered condition (Fig. 3b). In assay 3, inoculation of Wheatland resulted in a numerically larger mean lesion length under the water deficit condition (Wheatland  $P = 0.11$ ), while assay 4 did not indicate any differences ( $P \geq 0.22$ ). With regard to mean soil moisture, there was an effect for the assay, plant genetic background and water treatment ( $P < 0.01$ ), but not for inoculum ( $P = 0.92$ ). When Pearson correlation coefficients were determined, mean lesion length resulting from inoculation with *F. thapsinum* was weakly but significantly correlated with mean soil moisture content ( $r = -0.1818$ ;  $P = 0.04$ ), but not as a result of control inoculations ( $r = -0.0333$ ;  $P = 0.71$ ).



**Figure 2** Responses of wildtype sorghum plants, in the backgrounds RTx430 and Wheatland, to inoculation with (a) *Fusarium thapsinum* or (b) control, under water deficit (below 25% field capacity; left panel) or well-watered (field capacity; right panel) conditions, conducted in a greenhouse. The base of the stalk of each plant was wound-inoculated at anthesis with *F. thapsinum* or the control. Thirty-two days after inoculation, stalks were split to observe resulting lesions. Images were taken of plants in the same repetition.

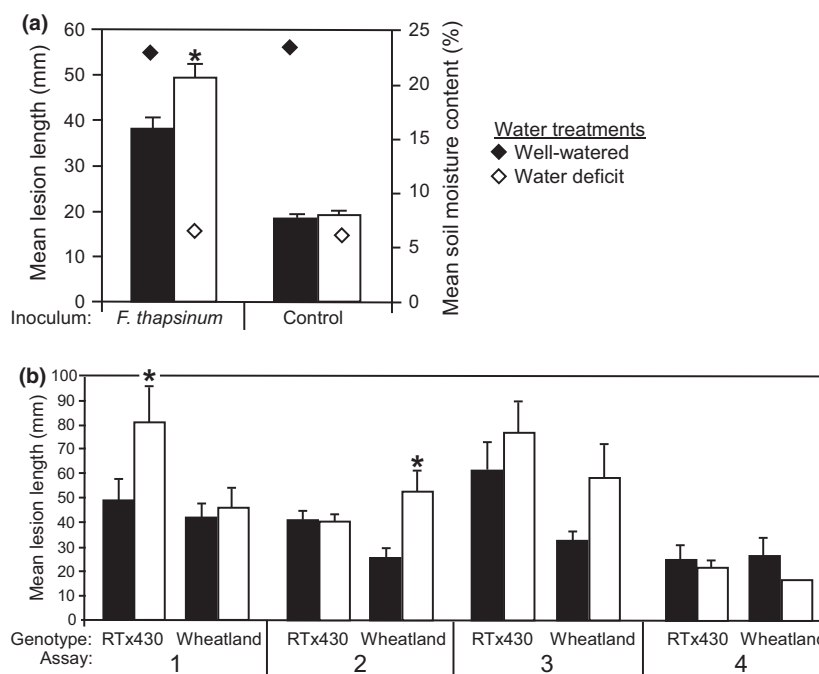
## Discussion

The results of this study provide strong evidence to support the first hypothesis that *bmr* lines are not more susceptible to fusarium stalk rot (*F. thapsinum*) inoculation in field studies. All of the *bmr* lines grown at each field had mean lesion lengths not significantly different or significantly smaller than corresponding wildtype. The RTx430 *bmr6 bmr12* double mutant plants grown at Mead had a significantly smaller mean lesion length than the corresponding wildtype, and this trend was also observed at Havelock ( $P = 0.10$ ). Previous greenhouse studies indicated there was no increased susceptibility of *bmr* lines as compared with wildtype for basal stalk inoculations with *F. thapsinum* (Funnell-Harris *et al.*, 2014). Therefore, results were consistent between previous greenhouse studies and the field results presented herein.

Wildtype, *bmr6* and *bmr12* plants in the genetic background RTx430, inoculated at Mead (irrigated) had significantly larger mean lesion lengths than corresponding *bmr* genotypes in Wheatland, but there were no significant differences in mean lesion lengths between RTx430 and Wheatland *bmr* genotypes when grown at Havelock (dryland). Mean lesion lengths resulting from inoculation of Wheatland plants at Mead by *F. thapsinum* were not significantly different from responses resulting on control inoculated plants, which suggests lower virulence of *F. thapsinum* on Wheatland plants grown at Mead, as opposed to those grown at Havelock. This result may have been due to reduced water stress and allowed Wheatland plants to resist the stalk pathogen (Bramel-Cox *et al.*, 1988; Tesso *et al.*, 2004).

Widely held concepts of the role of lignin as a barrier or induced inhibitory factor to pathogen invasion had been supported by previous research (Nicholson & Hammerschmidt, 1992; Guo *et al.*, 2016). The *bmr6* and *bmr12* mutations are in two genes that encode two enzymes in monolignol biosynthesis, the pathway that produces the three major cinnamyl alcohols (*p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) polymerized into lignin within the cell wall (Bout & Vermerris, 2003; Sattler *et al.*, 2009, 2012). *Bmr6* encodes a cinnamyl alcohol dehydrogenase, which acts at the last step in monolignol biosynthesis that produces the three cinnamyl alcohols. *Bmr12* encodes a caffeic acid *O*-methyltransferase, which acts at the second-to-last step of the branch that produces sinapyl alcohol. Results of response of sorghum pathogens to *bmr* lines and to phenolic compounds accumulating in *bmr* sorghum have suggested that changes in cell-wall-bound phenolic compounds from monolignol biosynthesis or compounds from other phenylpropanoid pathways such as flavonoids or salicylic acid associated with defence compounds or signalling may result in *bmr* plants being more resistant than the wildtype (Kamphius *et al.*, 2012), rather than free phenolic compounds with antimicrobial activity (Funnell-Harris *et al.*, 2017a). The flavonoid pathway in sorghum yields 3-deoxyanthocyanidin phytoalexins (Snyder & Nicholson, 1990), but other compounds derived from flavonoid biosynthesis may also be involved in inhibiting pathogens or pests. Additionally, it was recently shown that induction of the monolignol biosynthesis pathway in sorghum, by up-regulation of a Myb transcription factor (*SbMyb60*), could also result in increased levels of cell-wall-bound phenolics, such as sinapic and syringic acids (Scully *et al.*, 2017).

Although direct comparisons between mean lesion lengths resulting from field inoculations with *F. thapsinum* could not be made, results indicated that plant genetic background and environment influenced



**Figure 3** Response of sorghum lines RTx430 and Wheatland to inoculation with *Fusarium thapsinum* under well-watered (field capacity) and water deficit (below 25% field capacity) conditions. The base of the stalk of each plant was wound-inoculated at anthesis with *F. thapsinum* or control. Thirty-two days after inoculation, stalks were split and lesions were measured. (a) Mean lesion lengths (bars) in millimetres and positive standard errors (SE) for lesions produced on fungus-infected (left) and control plants (right) across both cultivars and all assays, under well-watered (black bars) and water deficit (white bars) conditions. Mean soil moisture content (percent) is indicated with diamonds for well-watered (black) and water deficit (white) conditions. (b) Mean lesion lengths and SE for four individual assays for lesions resulting from *F. thapsinum* inoculations produced on RTx430 (left) and Wheatland (right) plants under well-watered and water deficit conditions. For all analyses, least squares means (LSM) and SE are reported. Asterisks indicate mean lesion length is significantly larger than that of the corresponding treatment ( $P \leq 0.05$ ). Positive SE  $> 0.10$  are shown.

responses to *F. thapsinum*, as has been previously observed with fusarium stalk rot of sorghum (Bramel-Cox & Claflin, 1989). The field experiments included both dryland and irrigated environments. However, it was unclear what effect reduced water had on the interaction with *bmr* lines or genetic background. Therefore, a greenhouse assay was designed to more directly measure responses of the plant genetic backgrounds to the same pathogen isolate under water deficit and well-watered conditions. The data collected did not support the second hypothesis that fusarium stalk rot disease development under drought stress conducted under greenhouse conditions will not result in increased susceptibility to the pathogen in the wildtype lines. Indeed, greenhouse inoculations with *F. thapsinum* under water deficit resulted in increased virulence of the pathogen. This response was clear across the two genetic backgrounds tested. It has previously been shown with field-grown corn that water deficit treatment can result in significantly increased plant temperatures (O'Neill et al., 2006). Additionally, it has also been shown that *F. proliferatum* and *F. verticillioides*, closely related to *F. thapsinum* (Funnell-Harris et al., 2017b), grew faster at increasing temperatures from 22 to 30 °C (Samapundo et al., 2005). Thus, a combination of

increased fungal growth at higher temperatures and applied drought stress may have resulted in increased lesion length under greenhouse conditions. Therefore, inoculation of sorghum with *Fusarium* spp. in the greenhouse may be a useful tool for screening for fusarium stalk rot resistance under water deficit conditions. However, in individual assays, an effect was not always evident, which was presumably due to varying greenhouse conditions throughout the year (Funnell-Harris et al., 2016), so it is recommended that at least two repetitions be conducted to assess effects of water treatment on response to fusarium stalk rot pathogens. The interaction of cultivar with water treatment, and the three-way interaction with water treatment and inocula were not significant, indicating that RTx430 and Wheatland respond similarly to water deficit. However, the interaction of *bmr* with water deficit has not yet been determined. The response of *bmr* lines to water deficit and stalk pathogens under controlled greenhouse conditions is currently being assessed.

In summary, under field conditions *bmr* plants were not more susceptible to *F. thapsinum* than their near-isogenic wildtype counterparts. This conclusion is based on environmental responses to *F. thapsinum*, as indicated by mean lesion length, by near-isogenic *bmr* and wildtype

lines in two genetic backgrounds, RTx430 and Wheatland, grown at two field locations (Mead-irrigated; Havelock-dryland). The plant genetic background RTx430 demonstrated stable responses across the two environments, which included the *bmr6 bmr12* double mutant line that had a significantly smaller mean lesion length when inoculated at Mead and a trend towards smaller lesion lengths at Havelock, as compared with wildtype plants. Meanwhile, responses of genetic background Wheatland tended to suggest reduced susceptibility under irrigated conditions (mean lesion lengths following *F. thapsinum* inoculations were not significantly different from controls at Mead) compared to dryland conditions at Havelock. Wildtype lines of the same genetic backgrounds grown in the greenhouse under controlled well-watered and water deficit conditions provided further evidence that drought stress can result in significantly greater responses to the stalk rot pathogen, *F. thapsinum*, as quantified by lesion length, and indicated that the two genetic backgrounds responded similarly to the water treatments. However, these responses could vary in individual assays, possibly due to changing environmental conditions throughout the year, which was also previously observed with this pathogen (Funnell-Harris *et al.*, 2016).

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

**Table S1.** Environmental conditions during sorghum growing seasons at Mead, NE (irrigated) and Havelock field (dryland), in Lincoln, Nebraska, USA during the 2014, 2015 and 2016 growing seasons.

**Table S2.** Planting, inoculation and lesion measurement dates for field study in which sorghum *brown midrib* (*bmr*)-6, *bmr12* and *bmr6 bmr12* double mutant, and near-isogenic wildtype lines were inoculated with the stalk pathogen *Fusarium thapsinum*.